AD_____

Award Number: DAMD17-99-1-9319

TITLE: TGF-β and Breast Cancer Induction

PRINCIPAL INVESTIGATOR: Branka Brukner Dabovic, Ph.D.
Daniel B. Rifkin

CONTRACTING ORGANIZATION: New York University School of Medicine
New York, New York 10016

REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | July 2002 | Annual Summary (1 Jul 99 - 30 Jun 02) |

**4. TITLE AND SUBTITLE**
TGF-β and Breast Cancer Induction

**5. FUNDING NUMBERS**
DAMD17-99-1-9319

**6. AUTHOR(S)**
Branka Brukner Dabovic, Ph.D.
Daniel B. Rifkin

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
New York University School of Medicine
New York, New York 10016

E-Mail: dabovb01@med.nyu.edu

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**



**11. SUPPLEMENTARY NOTES**
report contains color

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. Abstract** *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
We attempted to generate mice that produced active TGF-ß in the breast by the use of a transgene containing the WAP promoter driving the expression of a truncated form of the latent TGF-ß binding protein (LTBP). TGF-ß is normally produced in a latent form and is part of a complex consisting of TGF-ß, the TGF-ß propeptides and (LTBP). We had shown that the expression of this type of transgene using the K14 promoter resulted in the production of active TGF-ß in the skin and the suppression of growth. We hypothesized that mice expressing this transgene would produce active TGF-ß, which would block the growth of epithelial cells and suppress tumor induction if expressed in mice with a high incidence of mammary tumors. Mice were produced that expressed the appropriate transgene in the breast. However, no consistent effect on the development of the glandular system in the breast was observed at 9, 24 or 36 weeks of age. The reasons for this may relate to the limited temporal expression of the WAP promoter. The effect of the transgene in mice susceptible to mammary tumors was not tested because of the failure to observe an effect in control mammary gland development.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| breast cancer, TGF-β, LTBP | 15 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

Table of Contents

**TGF-ß and Breast Cancer Induction** **Brukner Dabovic, Branka**

**Annual Training Report - Summary**

## Introduction

The purpose of this project was to test whether the presentation of the cytokine transforming growth factor-ß could be manipulated in the breast in such a way that initiation and/or development of tumors was effected. The project took advantage of the observation that essentially all TGF-ß is produced in a latent form, as part of a tri-molecular complex consisting of TGF-ß, the TGF-ß propeptides, and the latent TGF-ß binding protein (LTBP) (Derynck et al., 1985; McMahon et al., 1996; Miller 1992). In order for TGF-ß to act, it must be liberated from the complex (Miyazono et al., 1993; Harpel et al., 2001). We had found previously that by over-expressing a truncated form of the LTBP, which we call ECR3E, in the skin, the secreted TGF-ß appears to be active rather than latent. We reasoned that this occurred because the ECR3E competed for the endogenous LTBP for binding to the TGF-ß propeptide and this truncated form of TGF-ß was activated more easily (Gleizes et al., 1996; McMahon et al., 1996). We proposed to produce mice that expressed this shortened form of LTBP in the breast by the use of appropriate promoters and determine if this resulted in diminished induction of tumorigenicity in the breast, as TGF-ß is a potent inhibitor of the progression of normal mammary epithelial cells through the cell cycle (Pierce et al., 1993).

## Body

The project had as the Statement of Work five research tasks plus two tasks related to the preparation and publication of the data. The following are the accomplishments according to the proposed tasks.

Task 1- Construction of expression vectors for truncated LTBP regulated by the MMTV and WAP promoters. We were successful in preparing the WAP transgene by the methods outlined in previous reports. We were, however, never able to prepare the MMTV construct. We used MMTV constructs obtained from three different labs, but we were never able to produce a stable construct for the transgene that contained both the

MMTV promoter and the ECR3E region of the LTBP cDNA. We assume that for unknown reasons the combination of MMTV and ECR3E sequences were toxic to the bacteria. Therefore, we continued with the analysis of the WAP transgene. The WAP transgene was shown to be correct by sequencing and expression analysis.

Task 2- Create transgenic mice that over-express the transgene and cross these mice to introduce the transgene into mice that have a high incidence of breast tumor production. Transgenic mice over expressing the WAP-ECR3E transgene were produced by microinjection of DNA into FVB blastocysts. Five lines of transgenic mice were established, the transmission of the transgene monitored and the three lines with the highest expression of the transgene chosen for further study. The expression of the transgene was monitored by western blotting of protein extracts from breast tissue of normal and transgenic animals. The next task was to cross the WAP- ECR3E animals with MMTV-121 mice. These mice high a rapid development of breast tumors with 100% penitrance. Unfortunately, we found that these mice were no longer available from the laboratory that developed them We were able to find a second strain of mice, MMTV-neu #202 mice, that were commercially available and that also develop breast tumors within 140 days with 100% penetrance. These animals were obtained and a breeding colony established and crossed to the WAP-ECR3E mice we had produced.

Task 3- Time-course of tumor development in mouse strains. We felt that in order for the transgene to have an effect on tumor development sufficient active TGF-ß would have to be produced so that an effect on normal duct development in the breast would be noticeable when the breast morphology was examined during the estrus cycle. We decided to monitor this parameter before embarking on the longer term tumor induction studies. To do this, mice determined to be in estrus were sacrificed by asphyxiation, the mammary glands removed, spread over a microscope slide and fixed overnight in Carnoy's fixative. The next day, the specimens were hydrated and stained overnight in

Carmin alum stain that stains the epithelial ducts. After staining the samples were dehydrated in ethanol, cleared in xylene and mounted with Paramout.

At 4 weeks the mammary glands from virgin wild type and transgenic animals did not show any significant difference in the appearance of ductal branching or alveoli formation. At this age the ductal system has just started to form and occupies only about 1/3 of the glad. We reasoned that as there had been only one cycle of expression, the ECR3E had only been expressed once and this may not have been sufficient to affect the tissue. Therefore, we examined the ductal systems in mice at 9 weeks, at which time the ductal system occupies about 2/3 of the fat pad of the mammary gland. At this time we noticed a difference in the number of alveoli formed in the transgenic animals compared to wild type animals. The number of alveoli was decreased compared to the wild type littermates. This result corresponded well with the effect of active TGF-ß on mammary epithelia previously published by other groups. Therefore, this result was encouraging with respect to potential effects of the transgene on tumor formation.

We next analyzed the glands from 6-month-old virgin mice. These glands showed no significant differences in the appearance of the ducts and alveoli compared to wild type animals. At this stage the ductal system occupies the whole fat pad and is well spread and branched. It is possible that at this point the differences are not noticeable because the gland has been fully developed for some time and can compensate for the effects seen at 9 weeks. Therefore, we also examined glands in the periods before and after 9 weeks, but we were unable to observe any significant and/or reproducible effect of the transgene on the epithelium.

Because the expression of WAP is high during lactation, we examined the organization of the mammary gland during lactation. Unfortunately, the large amount of milk obscured the staining pattern and mad e the analysis impossible. We, therefore, shifted to an examination of the glands during the period of involution. To do this animals were bred, allowed to nurse for one week, and the pups removed. The animals were sacrificed at various times there after and the glands removed and examined. In the

samples form the transgenic mice, no differences where observed compared to the normal mice undergoing involution of the breast.

Tasks 3, 4, and 5 were not performed as stated above due to insufficient data to indicate the transgenic system would work.

During the time period of this project, I also completed a second project in which I produced a null mutation for the LTBP-3 gene. This mouse has a number of phenotypes in bone, all of which are consistent with LTBP-3 being required for proper TGF-ß presentation and activation. This work was published- J.C.B. 156,2002, 227-232. Two other publications have been submitted or are in preparation on this work in which I was partially supported by the DOD.

With respect to training, I learned how to make transgenic animals and how to analyze the ductal development in the breast.

**Key Research Accomplishments**

- Production of the WAP-ECR3E transgene.
- Production of transgenic mice with WAP-ECR3E transgene.
- Analysis of mice expressing the transgene.
- Development of staging algorithm for breast tissue.

**Reportable Outcomes**

- The development of the WAP-ECR3E mice.
- The development of an algorithm for staging breast tissue with respect to the reproductive cycle.
- The publication of a paper on the LTBP-3 knockout mouse.

## Conclusions

From these experiments we concluded that the effects on the transgene were either minimal or non-reproducible. There appeared to be a slight affect on the degree of alveoli formed at 9 weeks, but this observation was difficult to reproduce and was not observed in all animals. It is possible that in order to block duct formation, the transgene must be expressed at an earlier time during the cycle. Therefore, the use of the WAP promoter may have restricted the transgene expression. The use of the MMTV promoter may have yielded a more significant effect. One significant problem that we encountered was that it was quite difficult to stage mice with respect to their estrus cycle. Because of this we performed a study to compare the histology of the mouse breast and compare this with the histology of the human breast to see if there were sufficient similarities so that the state of estrus could be determined by the histology of the breast. Our preliminary results indicate that this can be done. We are continuing these studies to verify our results. The ability of investigators to know from examination of breast tissue the reproductive state of the animal will potentially insure that proper sampling is achieved. This is ongoing. We did not examine the induction of tumors in mice that had been bred to contain the transgene and the breast tumor promoting gene as we felt that the lack of reproducible data on an effect of the transgene on the development of the ductal tissues in normal mice indicated that it would be hard to monitor such effects on tumor production.

## References

Derynck, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., and Sporn, M.B. (1985) Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature **316**, 701-705

Gleizes, P. -E., Beavis, R.C., Mazzieri, R., Shen, B., and Rifkin, D.B. (1996) Identification and characterization of an eight-cysteine repeat of the latent transforming growth factor-β binding protein that mediates bonding to the latent transforming growth factor-β1.

Harpel, J.G., Schultz-Cherry, S., Murphy-Ullrich, J.E., and Rifkin, D.B. (2001) Tamoxifin and estrogen effects on TGF-β formation: role of thrombospondin-1, αvβ3 and integrin associated protein. Biochem. Biophys. Res. Comm. **284**: 11-14.
J. Biol. Chem. **271**, 29891-29896.

McMahon, G.A., Dighnam, J.D., and Gentry, L.E. (1996) Structural characterization of the latent complex between transforming growth factor β1 and β1 latency associated peptide. Biochem. J. **313**, 343-351.

Miller (1992) Characterization of the binding of transforming factor-β1, -β2, and -β3 to recombinant β1-latency associated peptide. Mol. Endocrinol. **6**, 694-702.

Miyazono, K., Ichijo, H., and Heldin, C.H. (1993) Transforming growth factor-β latent forms, binding proteins, and receptors. Growth factors **8**, 11-22.
Pierce Jr., D.F., Johnson, M.D., Matsui, Y., Robinson, S.D., Gold, L.I., Purchio, A.F., Daniel, C.W., Hogan, B.L., and Moses, H.L. (1993) Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-β1. Genes & Develop. **7**, 2308-2317.

# Bone abnormalities in latent TGF-β binding protein (Ltbp)-3–null mice indicate a role for Ltbp-3 in modulating TGF-β bioavailability

**Branka Dabovic,[1] Yan Chen,[1] Cristina Colarossi,[1] Hiroto Obata,[1] Laura Zambuto,[1] Mary Ann Perle,[2] and Daniel B. Rifkin[1,3]**

[1]Department of Cell Biology, [2]Department of Pathology, and [3]Department of Medicine, New York University School of Medicine, New York, NY 10016

The TGF-βs are multifunctional proteins whose activities are believed to be controlled by interaction with the latent TGF-β binding proteins (LTBPs). In spite of substantial effort, the precise in vivo significance of this interaction remains unknown. To examine the role of the Ltbp-3, we made an *Ltbp*-3–null mutation in the mouse by gene targeting. Homozygous mutant animals develop cranio-facial malformations by day 10. At 2 mo, there is a pronounced rounding of the cranial vault, extension of the mandible beyond the maxilla, and kyphosis. Histological examination of the skulls from null animals revealed ossification of the synchondroses within 2 wk of birth, in contrast to the wild-type synchondroses, which never ossify. Between 6 and 9 mo of age, mutant animals also develop osteosclerosis and osteoarthritis. The pathological changes of the Ltbp-3–null mice are consistent with perturbed TGF-β signaling in the skull and long bones. These observations give support to the notion that LTBP-3 is important for the control of TGF-β action. Moreover, the results provide the first in vivo indication for a role of LTBP in modulating TGF-β bioavailability.

## Introduction

The latent TGF-β binding proteins (LTBPs)*-1–4 are matrix molecules composed of multiple (14–20) EGF-like domains and four domains containing eight cysteines (8-cys) that are specific for the LTBPs and the fibrillins (for review see Handford et al., 2000; Koli et al., 2001). The modular structure of Ltbp-3 is shown in Fig. 1 A (Yin et al., 1995). LTBP-1, 3, and 4 covalently bind latent TGF-β (Koli et al., 2001). The TGF-βs are 25-kd homodimeric cytokines derived by intracellular proteolytic processing of larger proproteins (Massague, 1998). However, once cleaved from the cytokine, the TGF-β propeptide, called the latency-associated peptide (LAP), remains noncovalently associated with the mature TGF-β after secretion (Koli et al., 2001). This complex of TGF-β and LAP, the small latent complex, is inactive, and the dissociation of TGF-β from LAP is a crucial regulatory step in TGF-β action. The small latent complex can form a large latent complex by the bonding of cysteines in the LAP with a pair of cysteines in the third 8-cys domain of LTBP (Gleizes et al., 1996; Saharinen et al., 1996). All three TGF-β isoforms bind to LTBPs-1, 3, or 4; however, neither LTBP-2 nor the fibrillins bind TGF-β (Saharinen and Keski-Oja, 2000).

The LTBPs have been proposed to have two functions: as structural components of the extracellular matrix (Dallas et al., 1995) and as modulators of TGF-β bioavailability (Koli et al., 2001). Experiments in culture have shown that the association of LTBP with latent TGF-β is important for at least two aspects of TGF-β biology. First, the binding of LTBP-1 to the small latent complex facilitates its folding and secretion (Miyazono et al., 1991). Second, LTBP-1 modulates latent TGF-β activation (Flaumenhaft et al., 1993; Nakajima et al., 1997; Gualandris et al., 2000). However, these studies have not provided direct proof for the physiological role of LTBP in modulating TGF-β activity.

The online version of this article contains supplemental material.

Address correspondence to B. Dabovic, Dept. of Cell Biology, New York University Medical School, 550 First Ave., New York, NY 10016. Tel.: (212) 263-5327. Fax: (212) 263-0595. E-mail: dabovb01@med.nyu.edu

H. Obata's current address is Dept. of Ophthalmology, Jichi Medical School, Tochigi, Japan 329-0498.

*Abbreviations used in this paper: Bsp, bone sialoprotein; Ihh, Indian hedgehog; LAP, latency-associated peptide; LTBP, latent TGF-β binding protein; PTHrP, parathyroid hormone–related protein; PTHrP-R, PTHrP receptor; RIIDN, dominant negative receptor II; TGF-β, transforming growth factor-β.

Key words: latent TGF-β binding protein; TGF-β; osteoarthritis; osteosclerosis; synchondroses

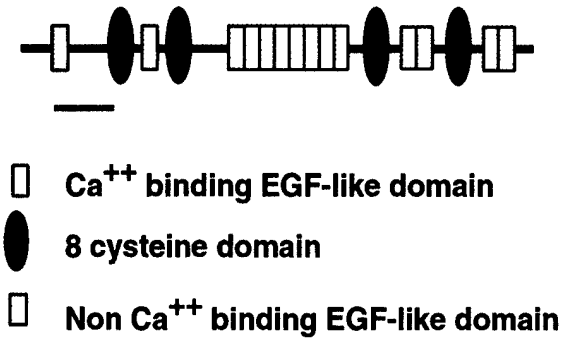



Figure 1. ***Ltbp-3* targeting strategy.** LTBP-3 protein structure. The region deleted in the targeted locus is underlined.

To address this point, we have generated Ltbp-3–null mice. These mice have an altered skull shape caused by the premature ossification of the cranial base synchondroses. In addition, there is enhanced accumulation of trabecular bone in the long bones and vertebrae and degeneration of the articular cartilage as the animals age. These phenotypic abnormalities are consistent with postulated roles of TGF-β in bone formation and homeostasis. As such, this report represents the first indication for physiological control of TGF-β activity by an LTBP.

## Results and discussion

### Generation of Ltbp-3–null mice

Ltbp-3–null mice were produced by gene targeting using a targeting vector to replace two exons containing a nonunit number of codons (Joyner, 1995), including bases 278–807 of the ORF with the neo$^r$ selectable marker (see online supplemental material, available at http://www.jcb.org/cgi/content/full/200111080/DC1, for details). These two exons code for the first EGF-like repeat, the pro-gly–rich region, and the beginning of the first 8-cys domain (Fig. 1). This deletion causes a frameshift in the ORF and premature termination of translation. Three clones (17, 22, and 25) with homologous recombination in the *Ltbp-3* gene were detected by Southern blot analysis (unpublished data), and two clones were used to produce chimeric animals. Ltbp-3–null animals were obtained by crossing heterozygous progeny of chimeric mice. Northern blot hybridization of lung RNA from null animals with a probe mapping 3′ from the deleted region showed the absence of the *Ltbp-3* transcript (Fig. 2 A). Therefore, we concluded that these mice were effectively Ltbp-3–null animals. Ltbp-3–null mice were born in the expected Mendelian ratio with no apparent defects. By day 10, null animals displayed a rounded head and shortened snout. X-ray radiography of 2-mo-old mutant mice revealed a domed skull, abnormal apposition of the upper and lower incisors, and curvature of the cervical and thoracic vertebrae (thoracic kyphosis) (Fig. 2 B).

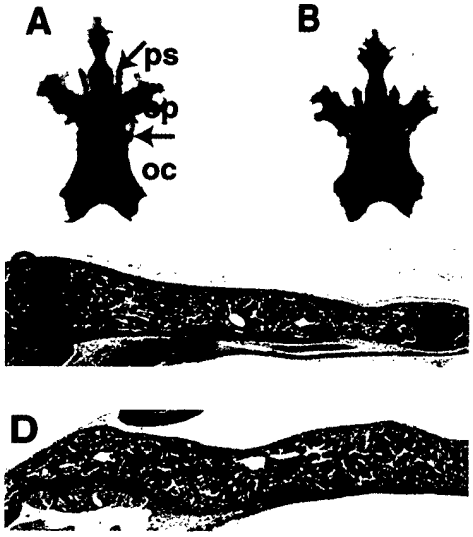
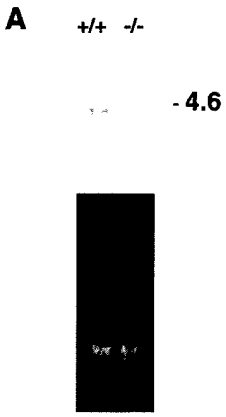



Figure 3. **Histology of wild-type and Ltbp-3–null skulls.** (A and B) Fusion of bones of the skull base in 3-wk-old Ltbp-3 mice. Whole mount skulls of wild-type (A) and mutant (B) animals were stained with alcian blue for cartilage and Alizarin red S for calcified bone, and skull bases were dissected. Arrows point to synchondroses between occipital (oc), sphenoid (sp) and presphenoid (ps) bones. (C and D). Histology of the skull base of 3-wk-old wild-type (C) and Ltbp-3–null (D) animals. Cartilage is stained red and bone blue. The basooccipital–basosphenoid synchondrosis is on the left.

### Craniofacial abnormalities

The appropriate anatomical development of the skull requires the coordinated growth of the membranous and endochondral bones to accommodate the increasing size of the brain. Therefore, the sutures between the bones of the cranial vault as well as growth plates in the skull base remain



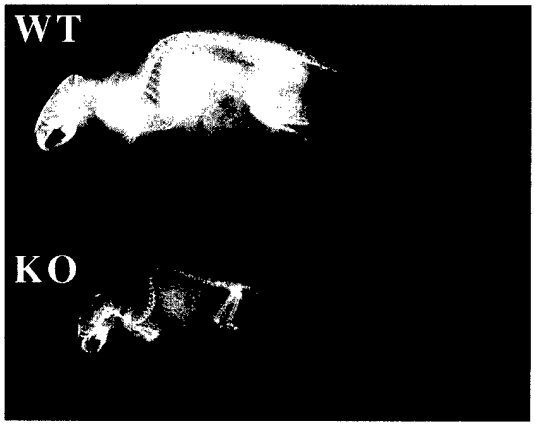




Figure 2. **Ltbp-3–null mouse.** (A) Detection of *Ltbp-3* mRNA in total lung RNA by Northern blot hybridization. (Top) Northern blot hybridization. +/+, Wild-type; −/−, *Ltbp-3*–null animal. (Bottom) ethidium bromide image of RNA before transfer. (B) X-ray radiography of 2-mo-old wild-type and Ltbp-3–null mice.

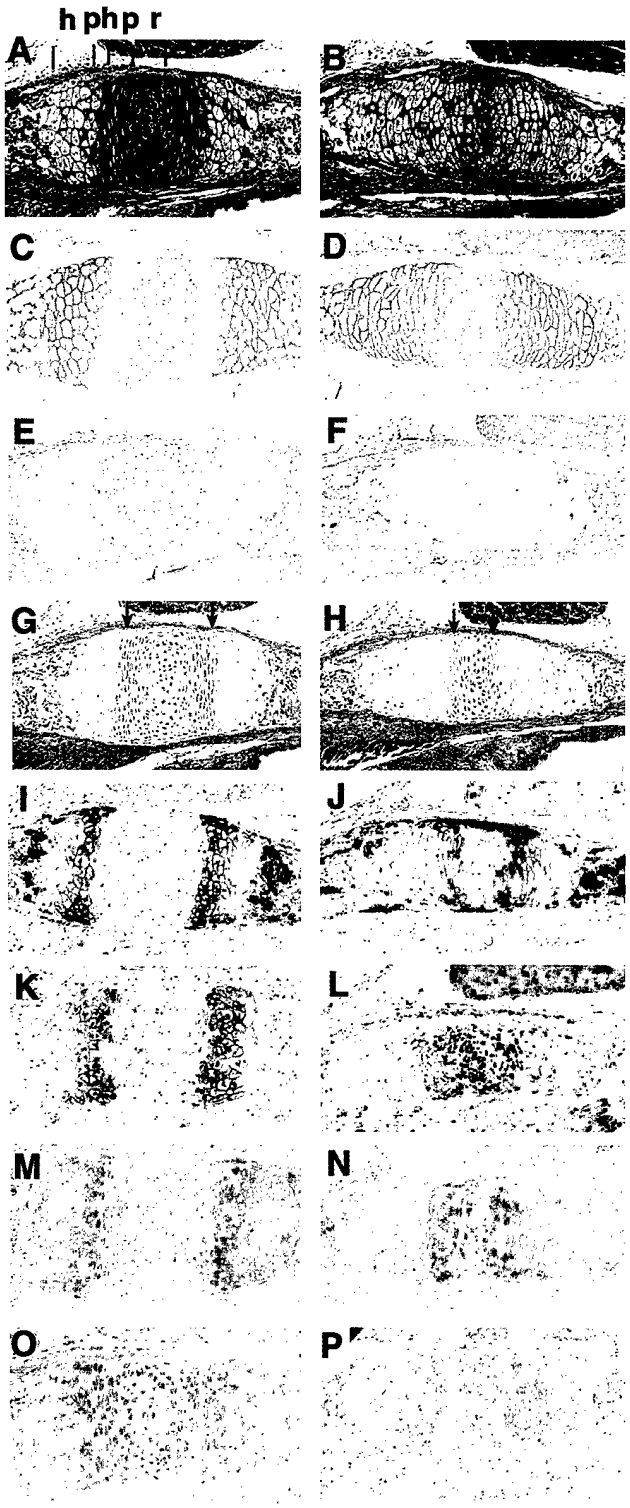



Figure 4. **The histological changes in basooccipital–basosphenoid synchondroses in 1.5-d-old wild-type and Ltbp-3–null animals.** (A and B) Weingart hematoxylin, Safranin O and Fast green staining. (A) Wild-type. (B) Mutant. Wider zone of hypertrophic chondrocytes in the mutant synchondrosis compared to the wild-type indicates more extensive differentiation. Cartilage is stained red. h, hypertrophic chondrocyte zone; r, resting chondrocyte zone; p, proliferating chondrocyte zone; ph, prehypertrophic chondrocyte zone; (C and D) Immunostaining for collagen X. (C) Wild-type. (D) Mutant. (E and F) Immunostaining for collagen II. (E) Wild-type. (F) Mutant. (G and H) Masson's trichrome staining for bone. More advanced bone fronts (blue) are apparent in mutant (H) versus wild-type (G) synchondrosis. Arrows point to fronts of the cortical basooccipital and basosphenoid bones. (I and J). In situ hybridization for bone sialoprotein 1 (*Bsp-1*). (I) Wild-type. (J) Mutant. (K and L) In situ hybridization for *Ihh*. (K) Wild-type. (L) Mutant. The strong signal in the middle of the mutant mouse synchondrosis (L) suggests that these chondrocytes are already committed to hypertrophic differentiation. (M and N) In situ hybridization for *PTH/PTHrP-R*. Expression pattern is less defined in the mutant animal sample (N) compared to the wild-type (M), and the transcript is detected through the central region of the synchondrosis. The intensity of the signal is similar in wild-type and in Ltbp-3–null samples. (O and P). In situ hybridization for *PTHrP*. In wild-type synchondroses (O) expression is apparent in proliferating chondrocytes and in lateral chondrocytes of the central region of the synchondrosis. The signal is absent in the resting chondrocytes in the center of synchondrosis. The intensity of the signal is decreased in Ltbp-3–null synchondrosis (P).

nonossified for an extensive period after birth. There are reports describing potential roles for TGF-βs in the differentiation of the membranous bones of the skull (Opperman et al., 1999), but there is no information concerning TGF-β function in the biology of the bones of the skull base. Histological studies of the sutures in wild-type and Ltbp-3–null animals revealed no pathological synostosis, i.e. premature fusion of one or more of the cranial vault sutures (unpublished data). However, differential staining for cartilage and bone in whole mount preparations, as well as histological analysis of the cranial base in 21 day old animals, revealed that the cartilaginous growth plates of the synchondroses were absent in mutant animals (Fig. 3, B and D), whereas the synchondroses were nonossified in wild-type littermates (Fig. 3, A and C).

Histologically, a synchondrosis resembles two opposed growth plates with a common zone of resting chondrocytes and separate zones of proliferating and hypertrophic chondrocytes. (Fig. 4 A). The earliest histological changes in the skull base of Ltbp-3–null animals were detected in the basooccipital–basosphenoid synchondrosis 1–2 d after birth. The overall structure of the synchondrosis was altered, as no distinguishable columns of proliferating chondrocytes were visible in the mutant synchondrosis (Fig. 4, compare A and B), and the zones of hypertrophic chondrocytes were wider. Collagen X, a marker for hypertrophic chondrocytes (Elima et al., 1993), was restricted to the ends of the synchondrosis in wild-type animals (Fig. 4 C), but was detected almost throughout the synchondrosis in Ltbp-3–null animals (Fig. 4 D). In addition, collagen type II, a marker for nonhypertrophic chondrocytes (Swalla et al., 1988), was present in the central zone of wild-type synchondrosis, but was absent from that of Ltbp-3– null animals (Fig. 4, E and F). The distance between the cortical bone fronts was smaller in the null animals compared to wild-type littermates as visualized by Masson's trichrome staining, which stains bone blue (Fig. 4, G and H). The ectopic ossification in mutant synchondrosis was also clear from the expression of the bone sialoprotein (*Bsp)-1* gene, an osteoblast specific marker (Bianco et al., 1991), in the cells surrounding the synchondrosis (Fig. 4, I and J). Although the basooccipital–basosphenoid synchondrosis was obliterated by 3–5 d after birth, the first changes in the basosphenoid–presphenoid synchondrosis in the null animals were not seen until days 3–10 (unpublished data).

Therefore, premature closure of the synchondroses in Ltbp-3–null mice is responsible for the observed craniofacial malformations. The failure to extend the presphenoid, basosphenoid, and the basooccipital bones results in a shortened cranial base. To accommodate the growing brain volume, the membranous bones of the vault expand outward and upward creating a domed skull. The anterior displacement of the foramen magnum may cause the development of the kyphosis, as the spinal column must be realigned with the overall body axis. The shortening of the cranial base also causes the alteration in the apposition of the incisors.

The wider zones of hypertrophic chondrocytes, as well as more advanced bone fronts in the Ltbp-3–null mice, resembled certain changes observed in animals deficient for the expression of parathyroid-hormone related protein (PTHrP) or its receptor PTH/PTHrP-R (for review see Karsenty, 2001). Vortkamp et al. (1996) proposed that chondrocyte differentiation is regulated by an inhibitory feedback loop in which Indian hedgehog (Ihh), produced by the most differentiated prehypertrophic and hypertrophic chondrocytes, stimulates the production of PTHrP by the periarticular cartilage and perichondrium, and PTHrP inhibits hypertrophic differentiation by interaction with its receptor on prehypertrophic chondrocytes (Chung et al., 2001). Therefore, we examined the expression of Ihh, PTHrP, and PTH/PTHrP-R in wild-type and mutant mice. In wild-type mice, Ihh was expressed by prehypertrophic and differentiating hypertrophic chondrocytes, whereas in null animals, Ihh expression was broader, indicating that chondrocytes in the central region of the synchondrosis were committed to hypertrophic differentiation (Fig. 4, K and L). In wild-type animals, PTH/PTHrP-R expression was detected in prehypertrophic chondrocytes, whereas in Ltbp-3–null animals, PTH/PTHrP-R transcripts were detected in the central region of the synchondrosis (Fig. 4, M and N). The expression pattern appeared less organized than that observed in wild-type synchondrosis, but the level of expression was similar as judged by the intensity of the staining. In sections probed for PTHrP, there was broad expression throughout the presumptive zone of proliferating chondrocytes, and the level of expression appeared lower in Ltbp-3–null animals compared to wild-type animals (Fig. 4, O and P). This decreased expression of PTHrP was also observed in the synchondroses of younger animals (see online supplemental material, available at http://www.jcb.org/cgi/content/full/200111080/DC1). A decreased level of PTHrP in Ltbp-3–null mice would allow more extensive chondrocyte differentiation and account for the more rapid ossification of the synchondroses.

The changes in Ltbp-3–null mice are consistent with previous reports describing a role for TGF-β; in regulating PTHrP expression, although we have been unable to detect differences with antibodies that recognize either active TGF-β or phosphorylated Smads. Pateder et al. (2001) and Serra et al. (1999) demonstrated in cell and organ culture that TGF-β stimulates PTHrP expression and inhibits hypertrophic differentiation. We infer that in Ltbp-3–null mice there is a deficit in TGF-β that results in decreased PTHrP expression and early differentiation of the synchondroses, ectopic ossification, and synostosis. The decrease in PTHrP

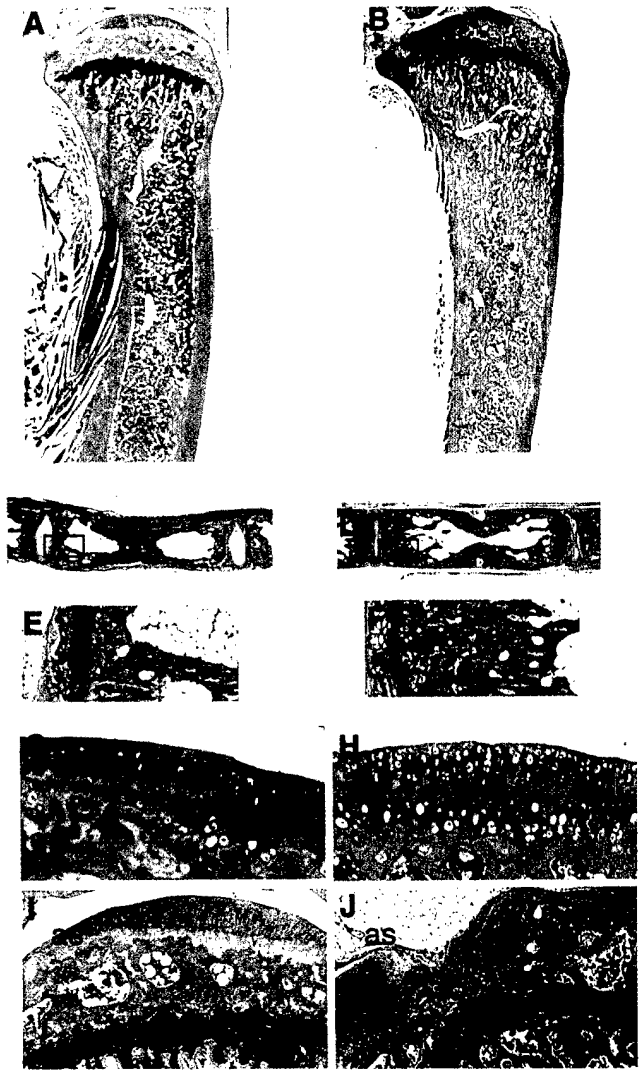



Figure 5. **Changes in the structure of long bones in Ltbp 3 mice.** (A–F) Mid-sagittal sections of tibiae (A and B) and caudal vertebrae (C–F) of 9-mo-old wild-type (A, C, and E) and mutant (B, D, and F) animals stained with Weingart hematoxylin/Safranin O/Fast green. (E and F) Higher (5×) magnification of the regions boxed in C and D. (G and H) Mid-sagittal sections of the articular region of proximal tibiae in 6-mo-old wild-type (E) and mutant mice (F). (I and J). Mid-sagittal section of epiphyses of 9-mo-old wild-type and Ltbp-3–null mice. ac, articular cartilage; as, articular surface; gp, growth plate; arrowheads, hypertrophic chondrocytes.

coincident with Ihh expression in the central zone of the synchondroses suggests that TGF-β acts before the induction of PTHrP expression and after Ihh expression.

## Defective bone remodeling

Because TGF-β has been shown to be important for long bone physiology, we examined the structure of the long bones of Ltbp-3–null mice to determine if other TGF-β-mediated functions were affected. By day 8 after birth, null animals showed growth retardation. The weight of adult Ltbp-3–null animals was 30–80% of sex-matched littermates, and the endochondral bones were shorter by ~10–25%. Histological analysis of the growth plates of tibiae, femora, and vertebrae from 1 d to 2-mo-old animals re-

vealed no obvious differences between Ltbp-3–null and wild-type littermates (unpublished data). Consistent with the report of Filvaroff et al. (1999), who expressed a type II TGF-β receptor with a truncated cytoplasmic domain (RIIDN) in osteoblasts and found age-dependent increases in trabecular bone mass, increased trabecular mass in long bones was observed at 3 mo of age in Ltbp-3–null mice with a body weight <60% of sex-matched wild-type littermates (unpublished data). This osteosclerosis was more pronounced in 6- and 9-mo-old mutant animals (Fig. 5, compare A and B). Staining for proteoglycans revealed unmineralized cores within the trabecular bones close to the growth plates, suggesting rapid extracellular matrix deposition and initiation of trabecular bone formation. The increased number of metaphysial trabeculae also suggested slow turnover. Similar changes occurred in the vertebrae (5, C–F). Interestingly, mutations in LAP cause Camurati–Engelmann syndrome, which is characterized by sclerosis and hyperosteosis (Janssens et al., 2000; Kinoshita et al., 2000), whereas transgenic animals overexpressing TGF-β2 in osteoblasts develop osteoporosis (Erlebacher and Derynck, 1996). Hence, we conclude that an Ltbp-3 defect mirrors long bone phenotypes caused by impaired TGF-β signaling.

The inhibition of TGF-β signaling has been shown to lead to periarticular cartilage terminal differentiation and ossification. Mice either expressing a TGF-β RIIDN in articular cartilage, synovium and periosteum/perichondrium (Serra et al., 1997), or deficient in Smad-3 (Yang et al., 2001) develop degenerative joint disease. Ltbp-3–null animals also develop progressive degeneration of articular cartilage resembling osteoarthritis. Histological analysis of the knee joints of mutant and wild-type littermates revealed pathological changes in the articular cartilage of 6-mo-old Ltbp-3–null animals: proteoglycan staining was decreased (compare Fig. 5, G and H) and hypertrophic chondrocytes were detected in the superficial layers of the articular cartilage (Fig. 5 H). In wild-type mice, the articular cartilage consisted almost exclusively of mature nonhypertrophic chondrocytes (Fig. 5 G). In mutant mice, at 9 mo, articular cartilage was absent, the articular surface appeared ossified and fibrotic (Fig. 5 J), and osteophytes were present (unpublished data). Similar changes were observed in the vertebral joints (Fig. 5, C–F). The degenerative changes in Ltbp-3–null joints present later (6–9 mo) than those in RIIDN transgenic and Smad-3–deficient animals. We believe that Ltbp-3 deficiency causes a more moderate decrease in TGF-β levels compared to the inhibition of TGF-β signaling in DNIIR and Smad-3–null animals.

## Summary

This report describes the phenotype of the Ltbp-3–null mouse: premature obliteration of synchondroses, osteosclerosis, and osteoarthritis. These manifestations are consistent with published evidence suggesting TGF-β involvement in bone remodeling and homeostasis. Therefore, we propose that Ltbp-3 regulates TGF-β bioavailability either by enhancing secretion of the TGF-β small latent complex or by participating in the activation of the latent TGF-β, as suggested from results obtained for LTBP-1 in cell culture (Miyazono et al., 1991; Flaumenhaft et al., 1993). Irrespective of the mechanism, the phenotypic changes in Ltbp-3–null mice are consistent with previous results describing a role for TGF-β in regulating PTHrP expression, as well as with the effects of impaired TGF-β signaling on bone physiology in vivo. Hence, this represents the first report providing genetic evidence in support of the role for LTBP in regulating TGF-β bioavailability. Experiments to determine the precise mechanism underlying these phenotypes are underway.

## Materials and methods

### Ltbp-3 gene targeting

Ltbp-3 gene targeting strategy, as well as the production and characterization of mice with a disrupted Ltbp-3 gene, are described in the online supplemental material (available at http://www.jcb.org/cgi/content/full/200111080/DC1). X-ray radiography was performed on anaesthetized animals using a Micro 50 (Microfocus Imaging) at 30 KV for 10 s.

### Histology and immunohistochemistry

Whole-skull staining with Alizarin red S and Alcian blue was performed as described (Lufkin et al., 1992). For histological analysis, samples were fixed overnight in 4% paraformaldehyde in PBS at 4°C, decalcified in 10% EDTA/2.5% paraformaldehyde in PBS for 7–14 d at 4°C, dehydrated through an ethanol series, cleared in xylene, and embedded in paraffin. 5-μm sections were stained with either Masson's Trichrome Stain or Weingart's hematoxilin/Fast Green/Safranin O (Luna, 1992) for bone and cartilage. Collagen II was detected using mouse monoclonal 2B1.5 antibody (NeoMarkers) and M.O.M. Kit (Vector) and collagen X using rabbit antiserum pXNC1-8, a gift from G. Lunstrum (Shriners Hospital for Children, Portland, OR) and the Vector Elite Kit.

### In situ hybridization

RNA probes were prepared using DIG RNA labeling kit (Roche). The probes used were *Bsp-1*, a gift from I. Thesleff (University of Helsinki, Helsinki, Finland), *Ihh*, a gift from A. McMahon (Harvard University, Cambridge, MA), PTHrP-R, gift of A. Broadus (Yale University Medical School, New Haven, CT), and PTHrP, a gift of H. Kronenberg (Massachusetts General Hospital, Boston, MA). In situ hybridization was performed as described (Wassarman et al., 1997).

### Online supplemental material

Included in the online supplemental materials (available at http://www.jcb.org/cgi/content/full/200111080/DC1) are details of the targeting strategy for production of *Ltbp-3* disruption and characterization of the genotypes of the mutant mice (Fig. S1), a picture of in situ hybridization of embryos probed for *Ltbp-3* expression (Fig. S2), and an illustration of PTHrP expression in the synchondroses of newborn animals (Fig. S3).

The authors would like to thank J. Annes, B. Arias, E. Arteaga-Solis, A. Auerbach, A. Joyner, S. Ortega, and M. Vassallo for assistance.

This work was supported by grants CA78422, CA34282, and DE13742 from the National Institutes of Health, and from the National Marfan Foundation to D.B. Rifkin. B. Dabovic was supported by Department of Defense grant DAMD 17-99-1-9319. H. Obata was supported by a Bausch and Lomb Overseas Research Fellowship.

Submitted: 26 November 2001
Revised: 13 December 2001
Accepted: 14 December 2001

## References

Bianco, P., L.W. Fisher, M.F. Young, J.D. Termine, and P.G. Robey. 1991. Expression of bone sialoprotein (BSP) in developing human tissues. *Calcif. Tissue Int.* 49:421–426.

Chung, U.I., E. Schipani, A.P. McMahon, and H.M. Kronenberg. 2001. Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. *J. Clin. Invest.* 107:295–304.

Dallas, S.L., K. Miyazono, T.M. Skerry, G.R. Mundy, and L.F. Bonewald. 1995.

Dual role for the latent transforming growth factor-beta binding protein in storage of latent TGF-beta in the extracellular matrix and as a structural matrix protein. *J. Cell Biol.* 131:539–549.

Elima, K., I. Eerola, R. Rosati, M. Metsaranta, S. Garofalo, M. Perala, B. De Crombrugghe, and E. Vuorio. 1993. The mouse collagen X gene: complete nucleotide sequence, exon structure and expression pattern. *Biochem. J.* 289: 247–253.

Erlebacher, A., and R. Derynck. 1996. Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype. *J. Cell Biol.* 132:195–210.

Filvaroff, E., A. Erlebacher, J.-Q. Ye, S.E. Gitelman, J. Lotz, M. Heillman, and R. Derynck. 1999. Inhibition of TGF-β receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass. *Development.* 126:4267–4279.

Flaumenhaft, R., M. Abe, Y. Sato, K. Miyazono, J. Harpel, C.H. Heldin, and D.B. Rifkin. 1993. Role of the latent TGF-beta binding protein in the activation of latent TGF-beta by co-cultures of endothelial and smooth muscle cells. *J. Cell Biol.* 120:995–1002.

Gleizes, P.E., R.C. Beavis, R. Mazzieri, B. Shen, and D.B. Rifkin. 1996. Identification and characterization of an eight-cysteine repeat of the latent transforming growth factor-beta binding protein-1 that mediates bonding to the latent transforming growth factor-beta1. *J. Biol. Chem.* 271:29891–29896.

Gualandris, A., J.P. Annes, M. Arese, I. Noguera, V. Jurukovski, and D.B. Rifkin. 2000. The latent transforming growth factor-beta-binding protein-1 promotes in vitro differentiation of embryonic stem cells into endothelium. *Mol. Biol. Cell.* 11:4295–4308.

Handford, P.A., A.K. Downing, D.P. Reinhardt, and L.Y. Sakai. 2000. Fibrillin: from domain structure to supramolecular assembly. *Matrix Biol.* 19:457–470.

Janssens, K., R. Gershoni-Baruch, N. Guañabens, N. Migone, S. Ralston, M. Bonduelle, W. Lissens, L.V. Maldergem, F. Vanhoencker, L. Verbruggen, and W.V. Hul. 2000. Mutations in the gene encoding the latency-associated peptide of TGF-β1 cause Camurati-Engelmann disease. *Nat. Genet.* 26: 273–275.

Joyner, A.L. 1995. Gene targeting, a practical approach. Oxford University Press, New York. 234 pp.

Karsenty, G. 2001. Chondrogenesis just ain't what it used to be. *J. Clin. Invest.* 107:405–407.

Kinoshita, A., T. Saito, H. Tomita, Y. Makita, K. Yoshida, M. Ghadami, K. Yamada, S. Kondo, S. Ikegawa, G. Nishimura, et al. 2000. Domain-specific mutations in TGFB-1 result in Camurati-Engelmann disease. *Nat. Genet.* 26:19–20.

Koli, K., J. Saharinen, M. Hyytiainen, C. Penttinen, and J. Keski-Oja. 2001. Latency, activation, and binding proteins of TGF-beta. *Microsc. Res. Tech.* 52: 354–362.

Lufkin, T., M. Mark, C.P. Hart, P. Dolle, M. LeMeur, and P. Chambon. 1992. Homeotic transformation of the occipital bones of the skull by ectopic expression of a homeobox gene. *Nature.* 359:835–841.

Luna, L. 1992. Histopathologic methods and color atlas of special stains and tissue artifacts. Johnson Pinus. Downers Grove, IL. 456 pp.

Massague, J. 1998. TGF-β signal transduction. *Annu. Rev. Biochem.* 67:753–791.

Miyazono, K., A. Olofsson, P. Colosetti, and C.-H. Heldin. 1991. A role of the latent TGF-β1-binding protein in the assembly and secretion of TGF-β1. *EMBO J.* 10:1091–1101.

Nakajima, Y., K. Miyazono, M. Kato, M. Takase, T. Yamagishi, and H. Nakamura. 1997. Extracellular fibrillar structure of latent TGF beta binding protein-1: role in TGF beta-dependent endothelial-mesenchymal transformation during endocardial cushion tissue formation in mouse embryonic heart. *J. Cell Biol.* 136:193–204.

Opperman, L.A., A. Chhabra, R.W. Cho, and R.C. Ogle. 1999. Cranial suture obliteration is induced by removal of transforming growth factor (TGF)-beta 3 activity and prevented by removal of TGF-beta 2 activity from fetal rat calvaria in vitro. *J. Craniofac. Genet. Dev. Biol.* 19:164–173.

Pateder, D.B., C.M. Ferguson, A.M. Ionescu, E.M. Schwarz, R.N. Rosier, J.E. Puzas, and R.J. O'Keefe. 2001. PTHrP expression in chick sternal chondrocytes is regulated by TGF-beta through Smad-mediated signaling. *J. Cell. Physiol.* 188:343–351.

Saharinen, J., and J. Keski-Oja. 2000. Specific sequence motif of 8-Cys repeats of TGF-beta binding proteins, LTBPs, creates a hydrophobic interaction surface for binding of small latent TGF-beta. *Mol. Biol. Cell.* 11:2691–2704.

Saharinen, J., J. Taipale, and J. Keski-Oja. 1996. Association of the small latent transforming growth factor-beta with an eight cysteine repeat of its binding protein LTBP-1. *EMBO J.* 15:245–253.

Serra, R., M. Johnson, E.H. Filvaroff, J. LaBorde, D.M. Sheehan, R. Derynck, and H.L. Moses. 1997. Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. *J. Cell Biol.* 139:541–552.

Serra, R., A. Karaplis, and P. Sohn. 1999. Parathyroid hormone-related peptide (PTHrP)-dependent and -independent effects of transforming growth factor β (TGF-β) on endochondral bone formation. *J. Cell Biol.* 145:783–794.

Swalla, B.J., W.B. Upholt, and M. Solursh. 1988. Analysis of type II collagen RNA localization in chick wing buds by in situ hybridization. *Dev. Biol.* 125:51–58.

Vortkamp, A., K. Lee, B. Lanske, G.V. Segre, H.M. Kronenberg, and C.J. Tabin. 1996. Regulation of rate of cartilage differentiation by indian hedgehob and PTH-related protein. *Science.* 273:613–622.

Wassarman, K.M., M. Lewandoski, K. Campbell, A.L. Joyner, J.L. Rubenstein, S. Martinez, and G.R. Martin. 1997. Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. *Development.* 124:2923–2934.

Yang, X., L. Chen, X. Xu, C. Li, C. Huang, and C.-X. Deng. 2001. TGF-β/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. *J. Cell Biol.* 153:35–46.

Yin, W., E. Smiley, J. Germiller, R.P. Mecham, J.B. Florer, R.J. Wenstrup, and J. Bonadio. 1995. Isolation of a novel latent transforming growth factor-β binding protein gene (LTBP-3). *J. Biol. Chem.* 270:10147–10160.